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Intra-cardiac and peripheral levels of biochemical markers of fibrosis in patients undergoing catheter ablation for atrial fibrillation.

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Introduction

There is convincing evidence from animal and human studies that myocardial fibrosis of the left atrium (LA) and/or the left ventricle (LV), is involved in the pathophysiology of atrial fibrillation (AF). The the role of LA fibrosis in particular is of interest as a possible target for identification of patients more likely to benefit from rhythm control strategies including ablation. [1-5] A number of methods of quantifying such fibrosis are available, including imaging techniques such as cardiovascular magnetic resonance imaging (CMRI) and echocardiography, and minimally invasive electrophysiological (EP) mapping. The role of circulating biochemical markers is also the subject of interest for predicting successful rhythm control, and numerous potential biomarkers have been studied, with mixed results.

In the context of catheter ablation of AF, the majority of studies assessing fibrosis biomarkers have tested peripheral levels. There is an assumption that peripheral levels of biomarkers will match intra-cardiac levels. However, this assumption has not been conclusively tested, so it is not clear whether intra-cardiac levels are indeed significantly different from peripheral levels. It is difficult to conclude, therefore, that any association between raised fibrosis markers and rhythm outcome is necessarily due to cardiac fibrosis, as opposed to systemic pathology.

This study was undertaken to compare peripheral levels of four biomarkers of fibrosis with intra-cardiac levels, to compare levels in AF patients with matched controls, and to compare the levels with left atrial fibrosis, assessed by EP mapping

during ablation. The biomarkers chosen were pro-collagen type III N-terminal pro-peptide (PIIINP), C-telopeptide of type I collagen (ICTP), fibroblast growth factor 23 (FGF-23) and galectin 3 (gal-3). In order to assess for any AF – specific association with these biomarkers, an age- and comorbidity-matched non-AF control group was also recruited for comparison.

Methods

Participants

Ethical approval was granted by the National Research Ethics Service Committee – Leeds West (ref. 13/YH/0349). Written informed consent was obtained from all patients. At a single institution, between September 2014 and August 2015, all consecutive patients (n=98) undergoing first-time left atrial ablation for paroxysmal, persistent, or long-standing-persistent AF were screened. Patients with systemic inflammatory disease, recent or active malignancy, severe kidney disease (eGFR < 30 ml/min/1.73m^2) or collagen disease were excluded, resulting in 95 participants. Two participants subsequently decided against ablation and withdrew themselves from the study, resulting in a final total of 93 participants in the AF ablation group. Recruitment of controls followed recruitment of AF patients. Patients attending cardiology clinics for non – AF related conditions were screened and selected to create a control group with matched overall population values for age, gender, left ventricular ejection fraction and comorbidities. Patients were excluded from the control group if they had any previously documented AF or other sustained arrhythmia of any cause, undiagnosed palpitations, or no documentation of sinus

rhythm. Other control group exclusion criteria were the same as for the AF group.

Echocardiography

All participants underwent trans-thoracic echocardiography prior to ablation by a single operator with over 5 years' experience. Images were obtained according to British Society of Echocardiography standard guidance. Atrial and ventricular volume measurements were obtained by Simpson's biplane method from apical 4-chamber and 2-chamber views. Antero-posterior left atrial diameter was measured in the 2D parasternal long-axis view. All atrial measurements were taken at the end of ventricular systole.

Serum analysis

Apart from blood sample collection, ablation procedures were carried out according to standard techniques. Whole blood was obtained from four sites per patient during the ablation procedure, but before any tissue ablation occurred. Femoral vein blood was aspirated via the femoral vein sheath. Intra-cardiac blood was aspirated via a long sheath placed in the right atrium, coronary sinus and, after trans-septal puncture, the left atrium. The first 10ml of aspirate from the sheath or catheter was discarded to ensure samples were not contaminated with saline previously used as a flush. Blood was transferred to sterile, non-pyrogenic serum separator tubes and allowed to clot for between 30 and 60 minutes. Tubes were then immediately centrifuged for 15 minutes at rcf x 1600g. Aliquots of the separated serum were then transferred to sterile, non-pyrogenic Eppendorf tubes and stored at -70^{o}C until analysis. Samples were then thawed prior to analysis, so underwent only one freeze-

thaw cycle. Blood samples from control patients were obtained from a peripheral vein using standard venepuncture equipment.

ELISA

Target biomarkers were analysed using commercially available enzyme-linked immunosorbent assay (ELISA) kits. PIIINP, FGF-23, and gal-3 were analysed using kits produced by Elabscience (Beijing, China). ICTP was analysed using kits produced by Cusabio Life Science (Wuhan, China). Kits were processed according to the manufacturer's instructions. Standards of known concentration and serum samples were tested in duplicate. Two wells per plate were used as 'blanks' to allow for background correction. Serum concentrations were extrapolated from optical density readings using a 4-parameter logistic curve derived from the standards. Inter- and intra-assay coefficients of variation were <15%. Lower limits of detection were; ICTP = 25ng/ml, gal-3 = 0.156ng/ml, FGF-23 = 15.625pg/ml, PIIINP = 23.438 pg/ml.

Electrophysiological mapping

Left atrial bipolar voltage maps were taken in all patients, irrespective of rhythm, after trans-septal puncture using a circular mapping catheter and either Carto (Biosense Webster) or NavX Velocity (St. Jude Medical) EP mapping systems. The minimum mapping time window for any electrode position was 2 seconds, in order to account for variation in voltage – particularly for those patient in AF during mapping. Data from these systems was then exported according to the manufacturer's instructions and reformatted to allow analysis on appropriate

software according to previously published methods.[6] Pulmonary veins, left atrial appendage and mitral valve surface were removed from the patient specific atrial anatomies generated during clinical mapping. The resulting shell was used to express the proportion of endocardial surface area which represented fibrotic LA tissue based on a voltage value between 0.2-0.5mV, as a percentage of the overall LA endocardial surface (not including the removed structures). *Figure 1* shows a representative voltage map.

Statistical analysis

Values are expressed as either median (interquartile range) for non-normally distributed data, or mean ± standard deviation for normally distributed data. A p-value of ≤0.05 was considered significant. Data were tested for normality using the Shapiro-Wilks test, and non-parametric data underwent logarithmic or square-root transformation if possible. For non-parametric data, comparison was made using Mann-Whitney test for independent samples, Wilcoxon's signed rank test for 2 paired samples, or Friedman's test for 3 or more paired samples. Where Friedman's test was significant, post-hoc Wilcoxon tests were carried out between the groups, with Bonferroni significance correction. Correlations were examined using Spearman's coefficient. For assessment of associations between baseline characteristics and biomarker levels, univariate linear regression analysis was performed, with mean biomarker levels across the sample sites for each participant as the dependent variable. Variables which were associated with a significance level of p=0.100 or less were then included in multivariate linear regression where the

variable data was appropriate for such analysis. Analysis was carried out using SPSS version 22 and Minitab version 17.

Results

Baseline characteristics and comparison with controls

Table 1 shows the baseline characteristics and comorbidities present within the study population, and comparison between characteristics of AF and control groups. There was no significant difference between the groups in age, gender, LV ejection fraction, or comorbidity. LA volume (but not diameter) was significantly higher in the AF group (p=0.007). There was no significant difference between levels of PIIINP, FGF-23 or gal-3 between the groups, however there was a significantly higher level of ICTP in the AF group (p=0.007).

Table 2 shows regression analysis of relationships between left atrial biomarker values and baseline characteristics within the AF ablation group.

After multivariate analysis, significant associations were found between body-mass index (BMI) and gal-3 (p<0.001), female gender and gal-3 (p<0.001), LV ejection fraction and ICTP (p=0.005), cerebrovascular disease and PIIINP (p<0.001), and time since AF diagnosis and PIIINP (p=0.003). Note that for LA voltage analysis, values are shown separately for patients in sinus rhythm and in AF during EP mapping. There were no associations between biomarker levels and LA voltage-defined fibrosis.

Comparison between blood sample sites

Figure 2 illustrates the distributions of individual biomarker values at each site. No significant difference was found between any of the sites for FGF-23 or PIIINP. Gal-3 levels were not available for RA and CS sites, but femoral levels were significantly higher than left atrial levels (p=0.001). Femoral ICTP was higher than LA and RA (p<0.001 for both), and CS ICTP was higher than LA (p=0.003) and RA (p<0.001). There was no significant difference between the atria, or between femoral and CS levels.

Discussion

In this study, our principal findings were that ICTP is higher in AF patients than in matched controls, that none of the studied biomarkers were associated with LA fibrosis assessed by voltage mapping, and for gal-3 and ICTP, intra-cardiac sampling may be necessary to assess their association with intra-cardiac processes. However, intra-cardiac sampling of FGF-23 or PIIINP gives no further information over peripheral sampling.

Atrial cardiomyopathy has recently been described expertly by Goette et al. in their exhaustive consensus statement.[7] Cardiac fibrosis is a hallmark of atrial cardiomyopathy and is characterised by an increase in the turnover of extra-cellular matrix. This matrix is principally comprised of type I and type III collagen, so PIIINP and ICTP are measurable products of this turnover. Both of these biomarkers have shown promise in the prediction of rhythm control success.[8, 9] Gal-3 has been shown to have direct and indirect effect on cardiac fibrosis and has been studied extensively

in the context of heart failure, in which it was found to be a relatively sensitive and specific marker of ventricular dysfunction, as well as mortality.[10-12]

FGF-23, principally studied in the context of its action on phosphate homeostasis in kidney disease and heart failure, has been associated with AF, increased LV mass, and cardiovascular death. [13-15] Results of studies assessing its association with incident AF are mixed. [16, 17] It has been shown to be required for cardiomyocyte proliferation and differentiation in early embryonic stages and this may be of interest in AF, as human cardiomyocytes *in vitro* have been shown to de-differentiate to a more primitive cell phenotype in cardiac fibrosis. [18] FGF-23 has been associated with increased atrial wall tension as manifest by raised NT-ProBNP levels, and it is unclear as to whether it has a direct effect on the atrial wall, or increases LA pressure by its effect on LV hypertrophy and fibrosis.

Participants and generalizability

As far as we are aware, this is the largest study that assesses the relationship between peripheral and intra-cardiac biochemical markers of fibrosis in an AF ablation population. The study population is representative of patients undergoing ablation for AF; despite screening of all 98 consecutive patients listed for AF ablation in a 12 month period, only 3 patients were excluded, and 2 withdrew. The results are therefore generalizable to the wider population of AF ablation patients.

Amongst the AF patient population as a whole, such patients represent a younger, healthier group with fewer comorbidities, with males being over-represented. As

such, the results may not be as generalizable to AF patient groups beyond those suitable for ablation. As expected, rates of comorbidity (e.g. cerebrovascular disease and renal impairment) were low in this population, so conclusions drawn about associations between the biomarkers and comorbidities should be drawn with caution.

It should also be noted that there is a large element of scatter amongst the biomarker readings as can be seen in figure 2 and the inter-quartile ranges of the biomakers. Despite this, however, statistically significant differences between the biomarker distributions could still be determined.

Relationship between intra-cardiac and femoral levels

Peripheral sampling of all four of these biomarkers proportionally represents their intra-cardiac levels. In the case of PIIINP and FGF-23, the lack of difference between peripheral and intra-cardiac levels suggests that these biomarkers may reflect systemic fibrosis (or other non-fibrotic processes in which they are involved). In the case of ICTP and gal-3, levels are higher peripherally. Therefore, it cannot be concluded that the heart is the main source of these substances in the bloodstream. Systemic rather than cardiac pathology may, therefore, be the principal driver behind any association between these biomarkers and successful treatment of AF. Such findings are in agreement with the study by Okumura et al., which showed no difference between intra-cardiac and femoral levels of their chosen inflammation and extra-cellular matrix turnover markers (which included ICTP) in 25 ablation patients. [8]

Intra-cardiac differences

PIIINP and FGF-23 were not significantly different between intra-cardiac sites. Therefore, any fibrosis present within the myocardium does not appear to contribute to a detectable increase of these compounds in the blood. However, ICTP was higher in the CS than either the LA or the RA. This suggests that ICTP enters the bloodstream from the myocardium at detectable levels, although, as discussed, this is masked in peripheral blood. This finding does not distinguish between ICTP arising from the ventricle or the atrium.

Relationship with baseline characteristics

In this study we found no evidence to support a relationship between the biomarkers and simple baseline echocardiographic markers of left atrial remodelling. Neither was there any relationship with AF classification, (paroxysmal vs. non-paroxysmal patients), however PIIINP was associated with duration of AF, measured by time since reported clinical diagnosis. PIIINP has been shown to have a complex relationship with AF incidence, with mixed results regarding its association with AF duration.[19] More work is required to understand the role PIIINP may play in relation to the temporal progression of the disease. The BMI and gender associations which we encountered with gal-3 have been previously described in other patient groups. [20, 21] A relationship between ICTP and ventricular function has also been described, to a much lesser extent. This study was not designed to examine such an association, and as only 5 patients had a reduced LVEF, further studies are required. Similarly, although an association between PIIINP and cerebrovascular disease was found in

this study, this was based on only 5 patients and should be interpreted with appropriate caution.

Relationship with LA voltage data

The presence of low voltage areas within the LA has been shown to be an independent predictor of AF recurrence after ablation, and there is evidence that these areas of low voltage are associated with fibrosis identified with MRI. [22, 23] Voltage criteria for identifying such areas are not universally established, however studies have addressed the issue. [22, 24] The definition of 'low voltage' in this study (0.2-0.5mV) is drawn from these studies. The lower value chosen was 0.2mV rather than 0mV to reduce artefact caused by poor contact between the mapping catheter and the endocardium. Assuming that this method is a consistent index of LA fibrosis, the results suggest that levels of these four biomarkers are not reflective of atrial fibrosis. For the biomarkers where there is some prior evidence of predictive value of arrhythmia recurrence (PIIINP, ICTP and gal-3), this may reflect their involvement in fibrosis elsewhere in the heart (e.g. the LV), or elsewhere in the body.

Implications

PIIINP and gal-3 levels have been associated with incidence of AF, but their use in the prediction of catheter ablation success has yielded mixed results.[12, 25, 26] This study may help to explain why this is the case, as it suggests that systemic (or at least non-atrial) factors may have the prominent role in their presence in the circulation, as evidenced by the lack of difference in matched controls. If cardiac fibrosis is the

critical component determining success in AF ablation, then such markers may have limited use in this role.

No studies have addressed the clinical utility of FGF-23 in predicting the success of AF ablation, and the findings presented here suggest such utility may be limited. On the other hand, ICTP does appear to be involved in local cardiac fibrosis and this may help to explain why it has shown more promise. In this study, however, we have shown that ICTP does not appear to be significantly associated with LA fibrosis. Therefore, it is potentially fibrosis elsewhere within the heart, most likely the LV, which the increased CS ICTP levels reflect.

Conclusion

ICTP levels are associated with the presence of AF in comparison with non-AF controls. PIIINP levels are associated with AF duration. None of ICTP, PIIINP, gal-3 or FGF-23 appear to reflect atrial fibrosis when assessed by voltage mapping criteria. Intra-cardiac sampling of FGF-23 or PIIINP gives no further information over peripheral sampling. For gal-3 and ICTP, intra-cardiac sampling may be necessary to assess their association with intra-cardiac processes.

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Disclosures

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Figure 1. Representative left atrial voltage map. Areas in red represent voltage of 0.2-0.5mV. Pulmonary veins and left atrial appendage were removed prior to analysis.

Figure 2. Individual value plots of biomarker levels at each sample site, with median values. Arrows on ICTP plot represent results of post-hoc testing of between-group differences.

Table 1. Participant characteristics and comparison with non – AF controls

Characteristic	Distribution		P value
	AF Group n= 93	Control group n = 36	

Age (years)	56.7 ± 11.9	60.7 ± 9.7)	0.073
BMI (kg/m^2)	29.7 ± 5.1	29.3 ± 5.1	0.679
Paroxysmal AF	63 (67.7)	-	-
Female gender	29 (31.2)	11 (30.1)	0.945
Hypertension	31 (33.3)	17 (47.2)	0.143
Diabetes Mellitus	9 (9.7)	5 (13.9)	0.490
Ischaemic Heart Disease	5 (5.4)	4 (11.1)	0.251
Chronic Kidney Disease	0 (0.0)	0 (0.0)	-
Cerebrovascular disease	5 (5.4)	5 (13.9)	0.105
CHA_2DS_2VASc Score **0** **1** **2** **3** **4** **5**	41 (44.1) 17 (18.3) 22 (23.7) 10 (10.8) 2 (2.2) 1 (1.1)	-	-

AADs*	**Amiodarone**	15 (16.1)	-	-
	Flecainide	16 (17.2)		
	Dispoyramide	1 (1.1)		
	Propafenone	1 (1.1)		
	Sotalol	2 (2.2)		
	None	58 (62.4)		
Rate-limiting drugs	**Beta-blocker**	54 (58.1)	-	-
	Ca^{2+} channel blocker	12 (12.9)		
	Digoxin	7 (7.5)		
	No drug	30 (32.2)		
	1 drug	56 (60.2)		
	2 drugs	6 (6.5)		
	3 drugs	1 (1.1)		
Total no. of drugs for rate/rhythm control of AF	**No drug**	16 (17.2)	-	-
	1 drug	51 (54.8)		
	2 drugs	23 (24.7)		
	3 drugs	3 (3.2)		

LA Volume (ml)	68.0 ± 22.5	56.1 ± 19.4	**0.007**
LA Diameter (mm)	40.5 ± 7.0	39.0 ± 5.7	0.263
Mean LA Pressure (mmHg)	11.0 (9.0)	-	
Mean RA Pressure (mmHg)	6.0 (6.0)	-	
LV end-diastolic volume (ml)	107.0 ± 29.6	106.9 ± 31.7	0.993
LV ejection fraction (%)	59.0 ± 7.1	57.3 ± 13.1	0.464
PIIINP (pg/ml)	60.8 (66.1)	54.6 (59.65)	0.749
ICTP (ng/ml)	330.1 (324.1)	221.2 (228.6)	**0.007**
FGF-23 (pg/ml)	39.7 (29.9)	37.4 (81.1)	0.334
Gal-3 (ng/ml)	27.7 (43.12)	22.0 (31.3)	0.323
Values = mean ± standard deviation, median (IQR) or frequency (%) as appropriate. AAD = anti-arrhythmic drug. BSA = body surface area. *No patients were on >1 AAD			

Table 2 Univariate and multivariate analysis of association between participant characteristics and biomarker levels

Characteristic	**PIIINP**		**ICTP**		**FGF-23**		**Gal-3**	
	Beta	P	Beta	p	Beta	p	Beta	p
Age (years)	0.033	0.838	-0.122	0.306	-0.188	0.339	0.211 *0.105*	0.059 *0.139*
BMI (kg/m^2)	0.043	0.789	0.112	0.344	-0.377 *-0.321*	0.048 *0.063*	0.429 *0.461*	0.000 ***<0.001***
Paroxysmal AF	0.078	0.625	0.047	0.692	0.399 *0.210*	0.035 *0.243*	-0.017	0.877

Time since AF diagnosis	0.343 *0.389*	0.020 ***0.003***	-0.062	0.610	-0.004	0.984	-0.161	0.873
Female gender	0.248 *-0.032*	0.114 *0.799*	-0.107	0.370	-0.214	0.274	0.484 *0.503*	0.000 ***<0.001***
Hypertension	0.172	0.277	-0.062	0.602	-0.053	0.789	0.095	0.397
Diabetes Mellitus	0.018	0.908	-0.184 *-0.171*	0.120 *0.166*	-0.054	0.787	0.084	0.458
Ischaemic Heart Disease	0.285 *0.097*	0.068 *0.449*	0.090	0.450	-0.025	0.898	-0.020	0.861
Myocardial infarction	-		-		-		-	
Chronic Kidney Disease	-		-		-		-	
Cerebrovascular disease	0.313 *0.511*	0.044 ***<0.001***	-0.024	0.838	-0.289	0.136	-0.064	0.567
CHA_2DS_2VASc Score	0.106	0.468	-0.197 *-0.037*	0.079 *0.775*	-0.197	0.315	0.245 *0.354*	0.028 *0.724*
Number of AF drugs	-0.061	0.677	-0.041	0.720	0.148	0.452	0.045	0.693

LA Volume / BSA (ml/m^2)	0.066	0.691	-0.105	0.397	-0.431 *-0.295*	0.032 *0.103*	-0.098	0.404
LA Diameter / BSA (mm/m^2)	0.185	0.252	-0.068	0.584	-0.327	0.103	-0.033	0.782
Mean LA Pressure (mmHg)	0.032	0.844	0.067	0.580	-0.302	0.126	0.209 *0.086*	0.067 *0.335*
Mean RA Pressure (mmHg)	0.031	0.851	-0.087	0.466	-0.462 *-0.295*	0.015 *0.077*	0.136	0.241
LV end-diastolic volume / BSA (ml/m^2)	0.102	0.573	0.005	0.967	-0.066	0.771	-0.098	0.404
LV ejection fraction %	0.140	0.431	-0.325 -0.339	0.001 ***0.012***	-0.050	0.827	0.035	0.778
LA voltage (% 0.2-0.5mV) (SR n=60)	0.364	0.080	0.003	0.988	0.104	0.761	0.080	0.652
LA voltage (% 0.2-0.5mV) (AF n=33)	0.171	0.867	-0.409	0.073	-0.576	0.135	0.311	0.131

Italics = results of multivariate analysis. ***Bold italics*** = significant association after multivariate analysis.

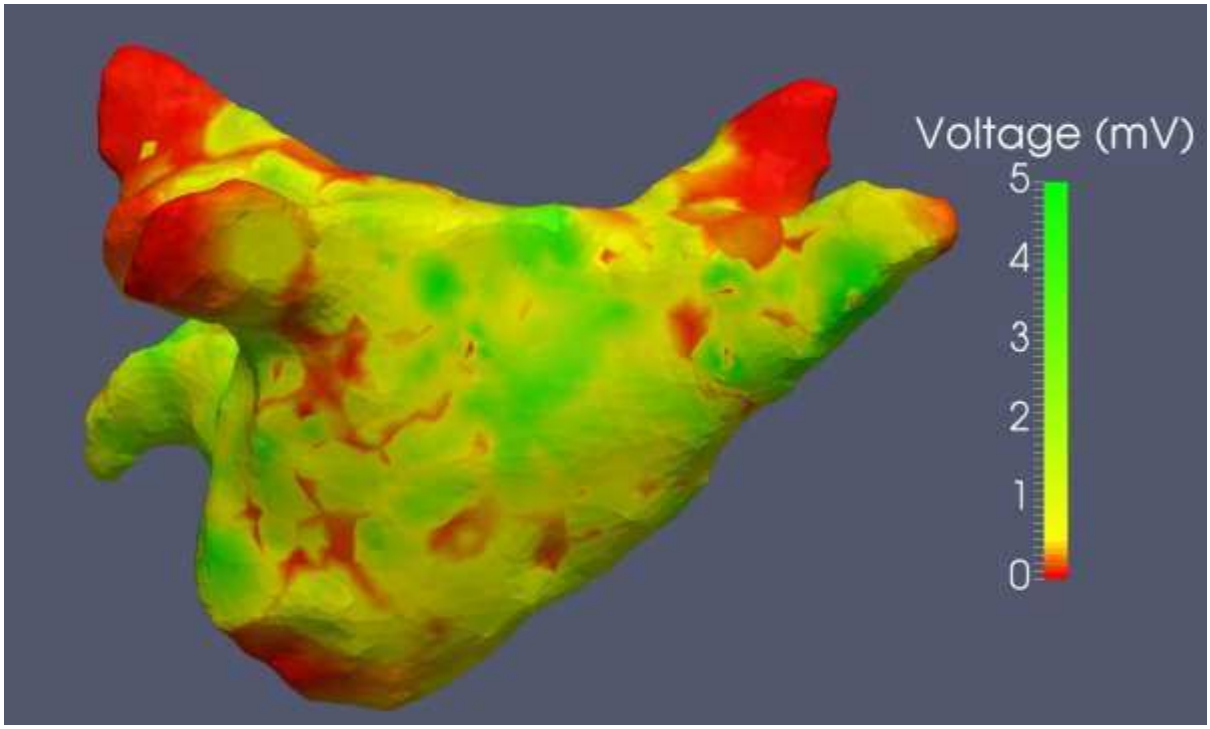

Figure 1

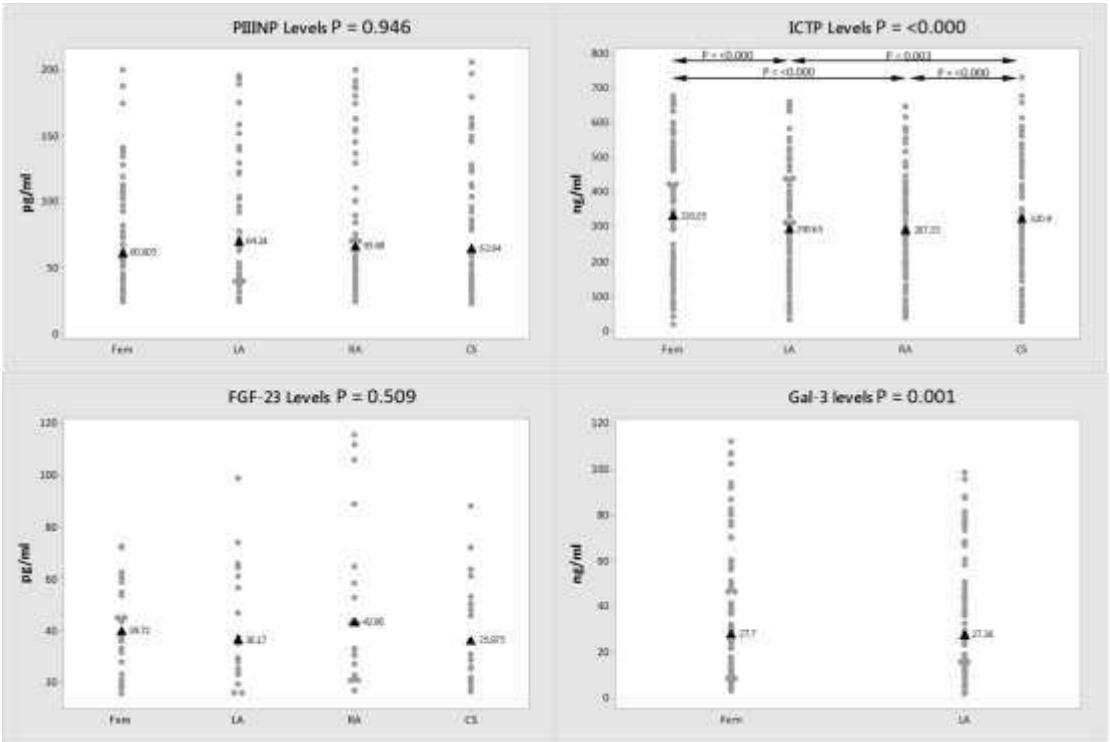

Figure 2